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INTRODUCTION

The general aim of this project is to determine the therapeutic efficacy and safety of treating prostate cancer by administering an Icon molecule that targets the tumor neovasculature for immunotherapy, using mouse models of mouse and human prostate cancer. The experiments reported in the following sections were done in immunocompetent mice carrying the mouse prostatic tumor RM-1, and in SCID mice carrying the human prostatic tumor C4-2. The specific aim is to compare several methods for delivering the Icon to these mice. In earlier published experiments, the Icon was encoded in an adenoviral vector that was injected intratumorally, creating *in vivo* factories of infected tumor cells that synthesize the Icon. The delivery methods studied for this project are as follows. (i) Icon-producer mammalian cells are entrapped in an alginate or thermosensitive gel, which is injected subcutaneously or intratumorally into the mice. (ii) Icon-producer mammalian cells are inserted into a chamber, which is implanted under the skin of the mice. (iii) Icon-producer mammalian cells are infected with an adenoviral vector encoding the Icon, and the infected cells are injected subcutaneously. (iv) The Icon is administered either by intratumoral injection of an adenoviral vector or intravenous injection of purified Icon protein.

The long-term aim is to develop and test different Icon delivery systems that could be adapted for use together or sequentially in clinical trials for prostate cancer immunotherapy.

Another aim of this project is to determine whether an Icon can be used for imaging the tumor vasculature as a diagnostic procedure for early stage prostate cancer. The initial experiments involve studying the biodistribution of radioiodine-labeled Icon in TRAMP mice that develop spontaneous prostatic tumors.

BODY

I. Assay for binding of the Icon to the mouse prostate cancer line RM-1 using Fluorescent Activated Cell Sorting (FACS)

RM-1 cells were grown in suspension in RPMI-1640 medium with 10% FBS. The cells were harvested by centrifugation, resuspended in FACS buffer (20 mM TrisHCl pH7.4, 150 mM NaCl, 5 mM CaCl₂, 1%BSA, 0.05% NaN₃) and divided into two samples, of which one was supplemented with 10 µg/ml of the mfVII-mFc Icon protein and the other used as a control. After incubation at 37°C for 35 min, the cells were washed once, resuspended in 100 µl FACS buffer containing 2 µl of anti-human IgG1-FITC conjugate, incubated on ice for 20 min and washed again. The washed cells were resuspended in 300 µl PBS containing 2 µg/ml propidium iodide and sorted on a FACS instrument.

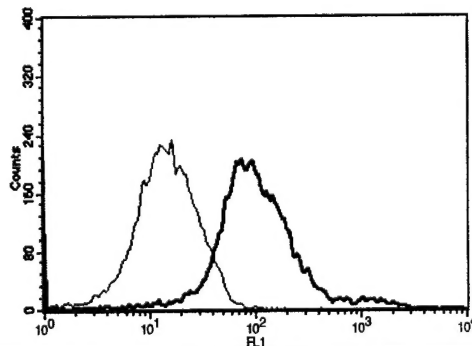


Fig. 1. FACS analysis for binding of mfVII-mFc Icon to mouse RM-1 cells.

The lighter curve on the left shows RM-1 control cells that were not incubated with the Icon, and the darker curve on the right shows the RM-1 cells that were incubated with the Icon. The displacement of the Icon-treated cells indicates that the Icon binds to virtually all of the RM-1 cells, presumably to tissue factor expressed on the cell surface.

II. Icon Producer Cells

The Icon producer cells were generated by two procedures, as follows. (i) One procedure involved transfecting Chinese Hamster Ovary (CHO) cells and Baby Hamster Kidney (BHK) cells with a cDNA encoding a secreted form of the mfVII-mFc Icon, and selecting stable transfectants that secrete a high level of Icon protein. (ii) Another procedure involved infecting human kidney 293 cells with a replication-incompetent adenoviral vector encoding a secreted form of the mfVII-hFc Icon. The infected 293 cells synthesize the encoded Icon protein and also produce infectious vector particles for 2-3 days, after which the cells lyse as the vector particles are released.

II-1. Containment and Maintenance of Icon-producer CHO and BHK Cells

To maintain viable producer cells *in vivo*, low molecular weight substances, such as oxygen, glucose and other nutrients, need be exchanged while immune cells and other factors that are toxic to the producer cell are excluded. A further consideration is that the Icon synthesized by the producer cells must be released into the systemic circulation.

The two types of containment devices are a gel matrix (1-5) and an enclosed semi-permeable chamber (6), as follows.

a. The gel matrix is composed of an alginate gel covered by an outer layer of poly-L-lysine, or a thermosensitive gel that is liquid at room temperature and forms a gel at physiologic temperature. The thermosensitive gels were produced in the laboratory of Dr. Anna Gutowska at the Pacific Northwest National Laboratory. For the alginate gel procedure, producer cells are trapped in the gel and injected as small gel particles. For the thermosensitive gel procedure, producer cells are mixed with the liquid gel and are trapped when the gel is injected and polymerizes. Both the alginate and thermosensitive gels allow molecules as large as the Icon (200 kD) to diffuse from the gel into the systemic circulation.

b. The chamber consists of a semi-permeable membrane enclosing a thin inner space. The producer cells are introduced inside the chamber, which is implanted under the skin. The membrane allows molecules as large as the Icon (about 200 kD) to diffuse from the chamber into the systemic circulation.

II-2. Safety and Efficacy of Gels Containing Icon-producer Cells

An essential requirement for using Icon-producer is that the cells should not escape from the containment device and colonize tissues in the organism. The following tests were designed to determine whether gels meet this requirement.

1. Alginate gel. An alginate gel containing Icon-producer CHO cells was injected s.c. into SCID mice. Examination of the mice on autopsy showed that the surface of the lungs had numerous colonies of cells, presumably initiated by CHO cells that had escaped from the gel. Because disseminated colonization by producer cells would pose an unacceptable risk for a therapeutic procedure, these experiments were discontinued.

2. Thermosensitive gel. We first compared in vitro the survival of CHO and BHK producer cells contained in three types of thermosensitive gels, called K-69W [linear P (NIPA-co-AAc)], K-69W [linear P (NIPA-co-AAc)], and K-63I [linear P (NIPA-co-AAc)]. The producer cells were mixed with the liquid gels in a 96-well plate at room temperature, and the plates were placed in a CO₂ incubator at 37C to initiate polymerization. Tissue culture medium was added to the polymerized gels, and the condition of the cells was monitored microscopically for one week. The cells survived only in the K-63I gel, which was used for the following experiment.

3. Intratumoral injections of a thermosensitive gel containing Icon-producing CHO cells into C57BL/6 mice carrying an RM-1 prostate tumor. RM-1 cells were injected subcutaneously into C57BL/6 mice, and after a skin tumor formed the mice were injected intratumorally with 50 ul of a 5% K-63I liquid gel (3 control mice) or with the gel containing 2.5×10^4 Icon-producing CHO cells (3 treated mice). Tumor volumes were monitored with a caliper. The red triangles show the volumes of the control tumors and the blue circles show the volumes of the Icon-treated tumors.

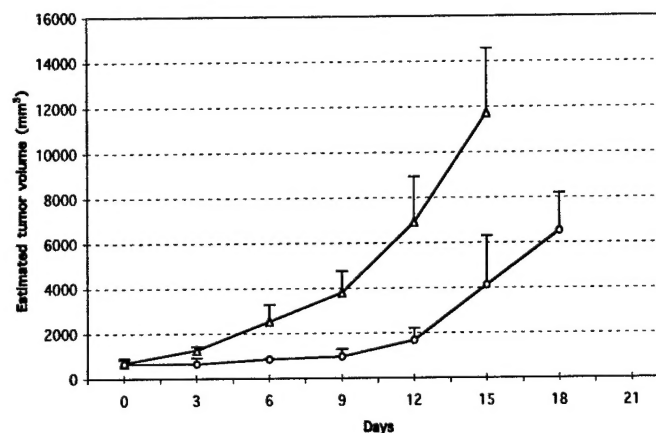


Fig. 2. Intratumoral injections of a thermosensitive gel containing Icon-producer CHO cells for immunotherapy of a mouse prostate tumor (RM-1) in C57BL/6 mice.

Examination of the mice at autopsy showed that the surface of the lungs had multiple nodules. Although the producer cells contained in the thermosensitive gel showed efficacy, nevertheless these experiments were discontinued because of concern that the CHO cells could escape and produce potentially metastatic nodules.

II-3. Safety and Efficacy of Injecting 293 cells Infected with an Adenoviral Vector encoding an Icon

Having found that neither an alginate gel nor a thermosensitive gel can prevent the escape of producer cells, we tested another type of producer cell that does not require containment because the cells are programmed to die in 3 days. The cells are the human kidney line 293 that functions as a helper cell for a non-replicative adenovirus. We first infected 293 cells with a non-replicative adenoviral vector encoding an Icon, and showed that the infected cells synthesize and secrete the Icon for about 2 days, after which progeny adenovirus are released and the cells lyse (7). The 293 cells were harvested on days 1, 2, or 3 after infection with the adenoviral vector and were analyzed by PAGE. The protein bands were transferred from the gel to a membrane, and the Icon bands were detected by incubating the membrane first with an anti-mouse Fc antibody followed by the ECL system (Amersham Pharmacia).

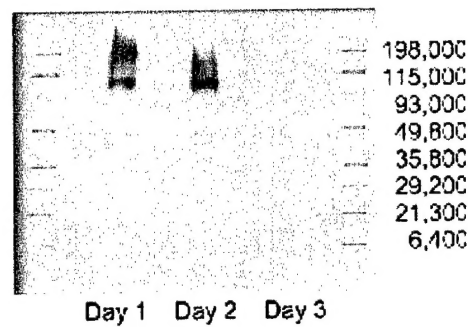


Fig. 3. Western assay for synthesis of the Icon by 293 cells infected with an adenoviral vector encoding the Icon.

To determine the biodistribution of the adenoviral infected 293 cells, the cells were infected with an adenoviral vector encoding the the green fluorescent protein GFP, and the infected cells were injected intradermally into mice. The results are shown in the following figure.

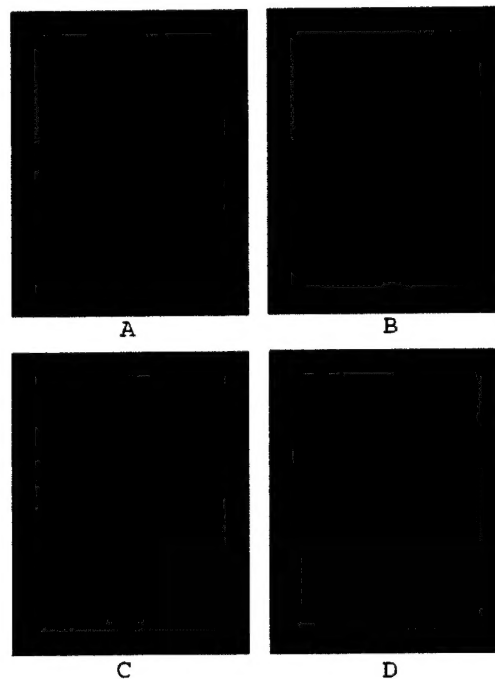


Fig. 4. Biodistribution of the adenoviral vector in mice after s.c. injection of 293 cells infected with the vector encoding GFP. After 3 days, the mice were sacrificed and frozen sections were prepared from the following tissues: (A) skin from the injected site; (B) skin from the contralateral flank that was not injected; (C) liver; (D) kidney. The sections were photographed with a fluorescent microscope at 200X magnification.

The 293 cells were infected with an adenoviral vector labeled with GFP cDNA inserted into the vector genome, and 5×10^6 infected cells were injected intradermally into the left rear flank of the mice. After 3 days, the mice were killed and frozen sections were prepared from the following tissues: (A) skin from the injected site; (B) skin from the right rear flank that was not injected; (C) liver; (D) kidney. The sections were

photographed with a fluorescent microscope at 200X magnification. The green spots in panel A are skin cells infected with the adenoviral vector produced by the 293 cells.

The next experiment was designed to test the safety and efficacy of injecting 293 cells encoding the mfVII-mFc Icon into mice carrying a mouse RM-1 prostatic tumor. A total of 5×10^5 RM-1 cells per mouse were injected s.c. in the rear right flank of C57BL/6 mice. When a skin tumor had formed, the mice were treated as follows. a) Control mice were injected with 293 cells infected with an adenoviral vector that did not encode the Icon. The vector was injected either intratumorally (\blacktriangle) or subcutaneously on the opposite side from the tumor (\triangle). b) Icon-treated mice were injected with 293 cells infected with an adenoviral vector encoding the Icon. The vector was injected either intratumorally (\bullet) or subcutaneously on the opposite side from the tumor (\circ). The number of mice in each group was 5. The number of intratumorally-injected 293 cells was 5×10^6 and the number of subcutaneously-injected 293 cells was 1×10^7 . The volume of the tumor was monitored by measurements with a caliper, and the results are shown in Figure 5.

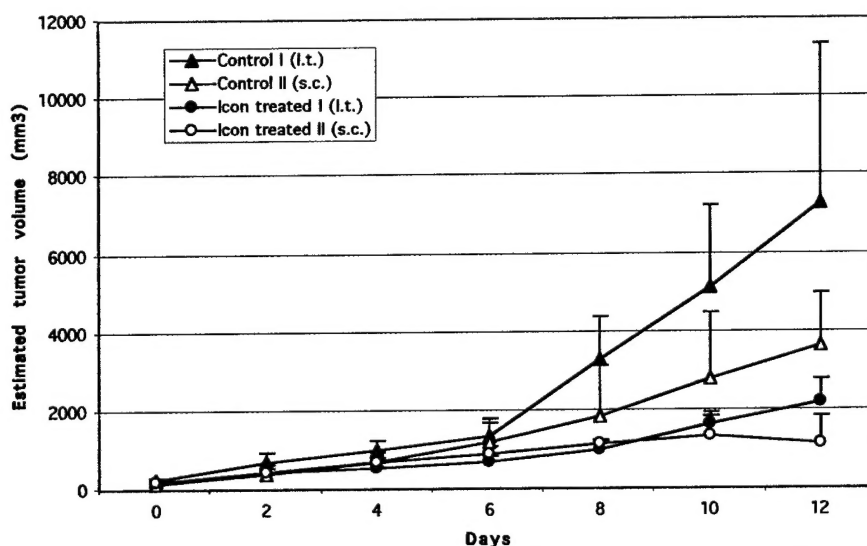


Fig. 5. 293 cells were first infected with an adenoviral vector encoding the Icon and then were injected either intratumorally or subcutaneously into C57BL/6 mice carrying prostate RM-1 tumors. The control mice were injected with 293 cells infected with a vector that did not encode the Icon. The tumor volumes were estimated from measurements with a caliper.

One conclusion is that intratumoral and subcutaneous injections of 293 cells infected with the vector encoding the Icon show significant efficacy in inducing regression of the RM-1 tumor. Another conclusion is that the procedure appears to be safe, since no cell nodules were detected with the 293 cells infected with the vector encoding the Icon.

II-4. Synthesis of the Icon in SCID Mice after Implantation of a Chamber Containing Icon Producer Cells

NIH-3T3 cells were transfected with the plasmid pcDNA3.1 encoding the mfVII-hFc Icon and a stable transfectant producing a high yield of the Icon was selected with G418. The figure 6 below shows a Coomassie-stained gel on the left and a Western blot stained with anti-human IgG Fc-HRP conjugate followed by an ECL-activated photographic film on the right.

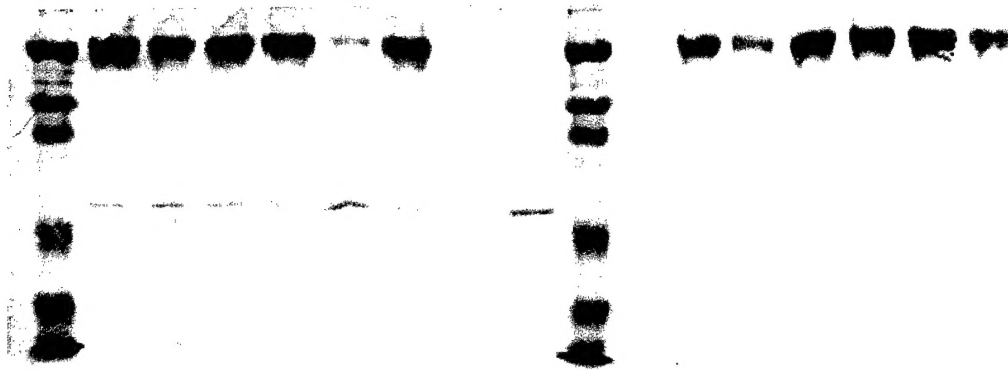
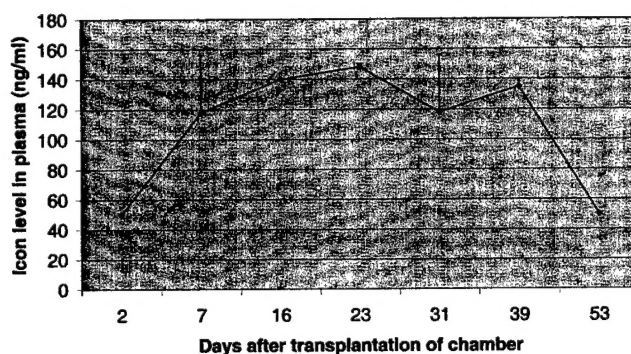


Fig. 6. Synthesis of the fVII/Fc Icon protein by NIH 3T3 Icon producer cells analyzed by SDS-PAGE (left panel) and by Western-blotting (right panel).

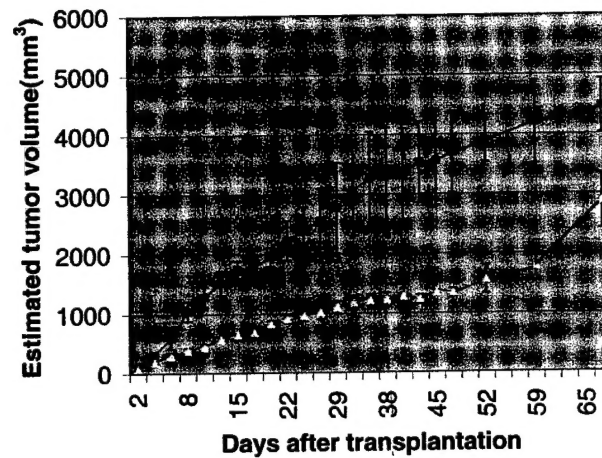
The transfectant was amplified and the cells inserted into a small TheraCyte chamber designed for use in mice (8-10), as follows. The chamber was moistened first with 95% ethanol followed by 70% ethanol for at least 2 hrs and rinsed with PBS. A suspension of 5×10^7 NIH-3T3 producer cells was inserted into the chamber, and the chamber was sealed with a silicone adhesive. Chambers containing the producer cells were implanted into CB-17/SCID mice carrying subcutaneous tumors under a skin flap that was closed by a suture. Blood samples were collected alternatively from each eye on days 0, 2, 4, 7, 10, 17, 24 and 39, and the plasma from the blood samples was tested for Icon protein by fluorescein-linked immunosorbent assay (Figure 7). The volume of the tumor was monitored by measurements with a caliper (Figure 8).

Figure 7



We conclude that the producer cells in the chamber secrete the Icon into the circulatory system for about 7 weeks.

Figure 8



We conclude that the Icon secreted by the Icon-producer cells in the chamber results in a significant inhibition of tumor growth for about 2 months. Further efficacy should be attainable by replacing the chamber with another chamber containing a fresh population of Icon-producer cells.

III. Efficacy and Safety of Delivering the Icon by Intratumoral Injections of an Adenoviral Vector and by Intravenous Injections of Purified Icon Protein

III-1. Intratumoral injections of an adenoviral vector for immunotherapy of human prostate cancer in SCID mice.

Prostate tumors were generated on one side by subcutaneous injection of the human prostate tumor line C4-2, which produces high titers of PSA and can metastasize to bone. The tumors were injected with an adenoviral vector encoding the Icon or with a control vector, and the tumor volume was monitored by mea such experiments are shown in Figure 9.

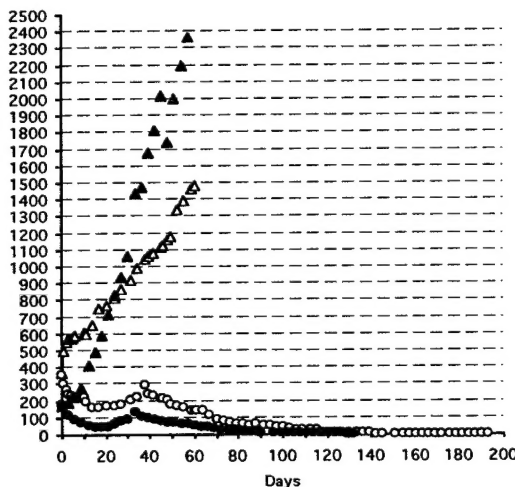


Fig. 9. Regression of a human prostatic tumor in SCID mice after intratumoral injections of an adenoviral vector encoding the mfVII/hFc icon. The first experiment involved 2 control mice (Δ) and 2 treated mice (\circ), and a second experiment involved 5 control mice (\blacktriangle) and 6 treated mice (\bullet). The mice were injected s.c. in one flank with the human prostatic cancer line C4-2. When the tumors had grown to an estimated volume of about 350 mm³ for the first experiment and about 180 mm³ for the second experiment, intratumoral injections were started (day 0). The dose/injection was 1×10^{10} VP. The mice received during the next 20 days a total of 7 injections for the first experiment and 6 injections for the second experiment either of the vector encoding the mfVII/hFc icon or the control vector. The tumors injected with the control vector grew rapidly, causing the mice to become seriously ill; all of the mice died between days 49 and 63. The tumors injected with the vector encoding the icon regressed initially, but started to grow again. The mice then received 3 injections for the first experiment and 4 injections for the second experiment from days 33 to 45, after which the tumors regressed and did not grow again for the duration of the experiments. At the end of the experiments only minute necrotic nodules of tumor tissue remained, similar to the tumor section shown below in Fig. 11.

III-2. To test the efficacy of the Icon for immunotherapy in a model of metastatic cancer.

Human prostate tumors were generated on two sides of the SCID mice and the adenoviral vector was injected into only one of the tumors, and the volumes of the tumors on both sides were monitored. The results are shown in Figure 10.

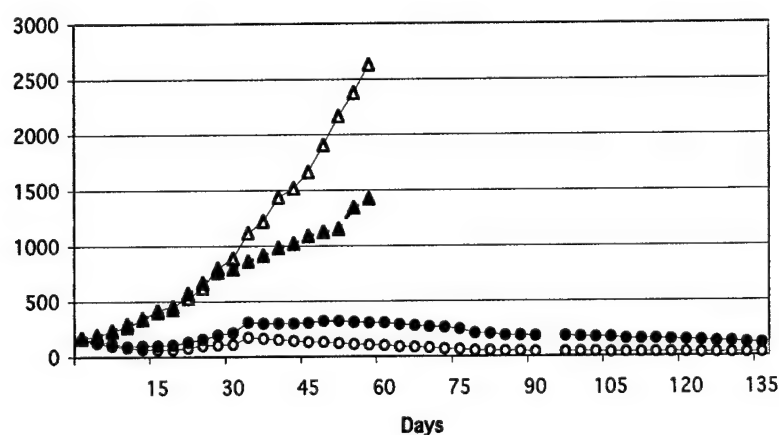


Fig. 10. Regression of two human prostatic tumors in SCID mice after intratumoral injections into one tumor of an adenoviral vector encoding the mfVII/hFc icon. The mice were injected s.c. in both rear flanks with the human prostatic cancer line C4-2. The resulting skin tumor on one flank was injected with the vector encoding the icon (4 mice) or with the control vector (4 mice) on days 0, 3, 6, 9, 12, 15, 33, 36, 39 and 42, while the tumor on the other flank remained uninjected. The dose was 1×10^{10} vector particles per injection. The control mice were euthanized on days 53 to 57 because the tumors had grown too large; the tumors in the icon-treated mice regressed and the mice were euthanized on day 138. \circ : Tumors injected with the vector encoding the icon. \bullet : Uninjected tumors in the Icon-treated mice. Δ : Tumors injected with the control vector. \blacktriangle : Uninjected tumors in the control mice.

The tumors from the experiment of Figure 10 were excised when the mice were autopsied, and the tumors were examined histologically as shown in Figure 11.



Fig. 11. Histology of the human prostatic tumors from the experiment described in Figure 10. The tumors were excised during necropsy on day 138 for the icon-treated mice and on day 57 for the control mice. The sections were fixed in formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Panel A: Tumor injected with the control vector, showing dense vascularization and viable tumor cells. Panel B: Injected tumor from the icon-treated mouse. Panel C: Uninjected tumor from the icon-treated mouse. Note that Panels B and C, and also the areas of the two sections not shown in the panels, do not contain viable tumor cells.

The C4-2 human prostate tumor line produces high serum titers of PSA (prostate-specific antigen), providing a sensitive indicator of viable tumor cells. The PSA titers in the serum of Icon-treated and control mice from Figures 9 and 10 are shown in Table 1.

Table 1. PSA titer in plasma of SCID mice after intratumoral injections of the adenoviral vector encoding the mfVII/hFc icon or the control adenoviral vector into a human prostatic tumor.

Experiment	Control mice			Icon- treated mice		
	Injected mouse	Sample day	PSA (ng/ml)	Injected mouse	Sample day	PSA (ng/ml)
Fig. 9 #1	1	60	194.4 \pm 6.3	1	142	0.6 \pm 0.6
	2	49	8.0 \pm 1.2 ^a	2	142	0.6 \pm 0.6
Fig. 9 #2	1	57	1891 \pm 10	1	105	0.6
	2	48	1739 \pm 37	2	105	0.0
	3	57	1240	3	105	24
	4	63	170	4	105	0.0
	5	63	314			
Fig. 10	1	57	371	1	105	0.6
	2	57	1504	2	105	0.4
	3	57	926 \pm 33			
	Uninjected mouse		0.6 \pm 0.6			

(a) The tumor in this mouse had extensive regions of necrotic cells, which could have accounted for the relatively low PSA titer.

We conclude from the results shown in Figures 9, 10 and 11 and Table 1 that intratumoral injections of an adenoviral vector encoding the Icon result in eradication of a human prostatic tumor xenograft without associated toxicity. The treated mice appeared healthy and tumor-free for at least 150 days after the last injection of the vector (see Figure 9).

III-3. Intravenous injections of purified Icon protein for immunotherapy of human prostate cancer in SCID mice.

Human prostate cancer LnCap cells were mixed with Matrigel and injected subcutaneously into 20 male CB-17/SCID mice. When tumor size reached about 160-190 mm³, Icon protein in PBS buffer was injected intravenously into 3 groups of mice with 5 mice in each group. The amount of protein injected in each group 0.2 ug, 2 ug and 20 ug, respectively, on day 0 and 8. A control group of 5 mice was injected with PBS buffer only. Tumor volumes were monitored by measurements with a caliper (Figure 12). At the end of experiment, the tumors were weighed (Figure 13). All of the organs and tissues in the mice were examined morphologically and appeared normal.

Figure 12

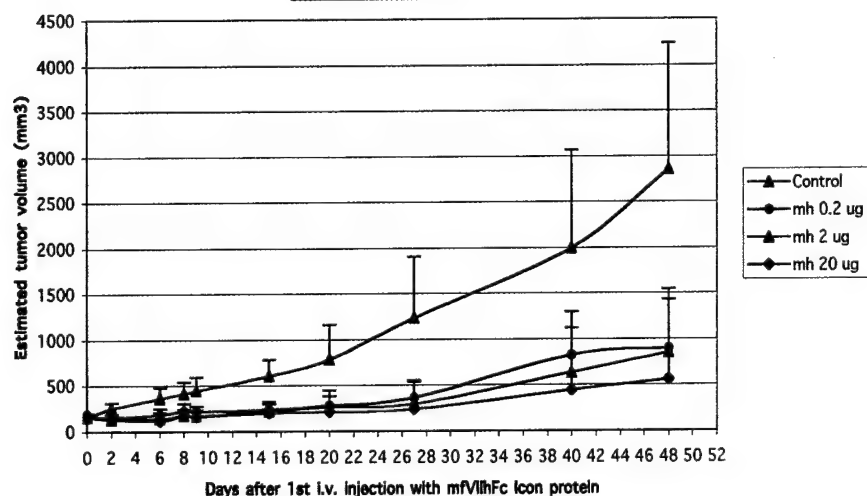
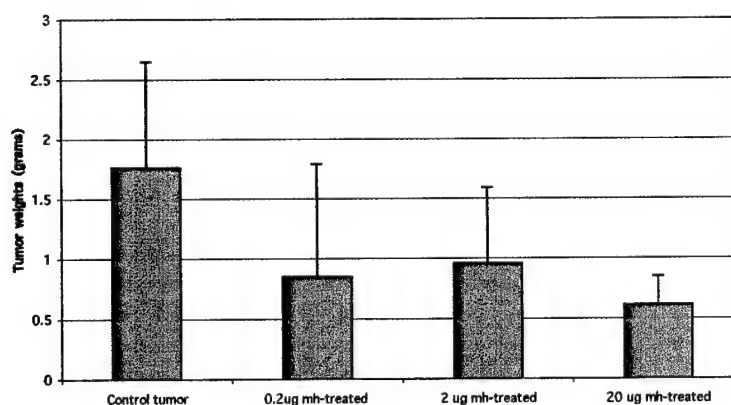


Figure 13



IV. Biodistribution of Icon Protein Injected Intravenously into a TRAMP Mouse

The TRAMP mouse genome contains a SV40 large-T-antigen gene driven by a prostate specific promoter, resulting in spontaneous arising prostatic tumors (11). The experiments with TRAMP mice were done in collaboration with Dr. Albert Deisseroth at the Sidney Kimmel Cancer Center in San Diego, CA. The Icon protein was labeled with ^{131}I using the chloramine-T method, and 10 micrograms of the labeled protein was injected into the tail vein of a male TRAMP mouse that had developed extensive intra-abdominal tumors. After sedation the mouse was placed in the supine position under a gamma emission scintigraphy pin hole camera, and the contours of the animal were defined by a radioactive marker. The biodistribution of the Icon is shown in the following figure 14.

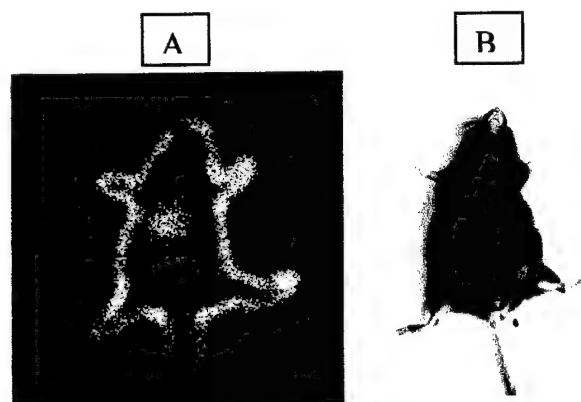


Fig. 14. Biodistribution of the ^{131}I -labeled Icon protein in a TRAMP mouse carrying spontaneous prostate tumors after intravenous injection of the radioiodinated Icon protein. Panel A shows an autoradiograph of the mouse, and Panel B shows a photograph of the mouse before autoradiography.

Panel B shows 2 lower tumors and 2 upper tumors. The lower tumors were cystic or semi-solid with necrotic fluid, and the upper tumors were hard and contained viable tumor cells. Panel A shows intense uptake in the region of the upper tumors. No involvement of the liver, lungs, spleen or kidney was evident on gross inspection. These results suggest that the Icon binds preferentially to tumors containing viable cells.

KEY RESEARCH ACCOMPLISHMENT

The overall aim of this project is to test different methods of delivering an Icon for efficacious and safe immunotherapy of prostatic tumors.

1. We tested the safety of an alginate gel containing Icon-producer CHO cells by subcutaneous injection of the gel into SCID mice. Examination of the mice on autopsy showed that the surface of the lungs had multiple sites with large masses of cells, presumably initiated by CHO cells that had escaped from the gel. Similar results were obtained with a thermosensitive gel. Because disseminated growths generated by producer cells that escape from the gel would be an unacceptable risk for a therapeutic procedure, the experiments were discontinued.

3. We tested the efficacy and safety of injecting 293 helper cells infected with an adenoviral vector encoding the Icon. The infected 293 cells produce and release vector particles and the encoded Icon. Intratumoral and subcutaneous injections of the infected 293 cells in C57BL/6 mice carrying a mouse prostatic tumor resulted in significant inhibition of tumor growth. The procedure also appeared to be safe, since there was no evidence of disseminated nodules derived from the 293 cells infected with the vector encoding the Icon. The advantage of using infected 293 cells is the production of additional vector particles *in vivo*, resulting in additional production of the Icon and enhanced efficacy.

4. We tested the efficacy and safety of inserting Icon-producer NIH 3T3 cells into a TheraCyte chamber and implanting the chamber under the skin of SCID mice carrying a human prostatic tumor. Synthesis of the Icon was detected in the blood for about 7 weeks, resulting in significant inhibition of tumor growth. The efficacy of this procedure could be extended for a longer period by replacing the chamber with a new chamber containing a fresh population of Icon-producer cells.

5. We tested the efficacy and safety of injecting intravenously purified Icon protein as compared to injecting intratumorally an adenoviral vector encoding the Icon. Both procedures showed impressive long-term efficacy against a human prostatic tumor xenograft in SCID mice without evidence of toxicity.

6. The potential use of the Icon for imaging tumors was tested by injecting radioiodine-labeled Icon intravenously into TRAMP mice containing spontaneous prostatic tumors and determining the biodistribution of the label. Preliminary data showed strong uptake of the Icon in tumors containing viable cells, suggesting that the Icon could be developed as a diagnostic probe for cancer.

REPORTABLE OUTCOMES

We intend to complete the studies described in this report and to publish the results.

CONCLUSIONS

1. Alginate gels and thermosensitive gels are not suitable for encapsulating Icon-producer cells because the cells can escape from the gel and form disseminated nodules.
2. Infecting 293 cells with an adenoviral vector results in amplified production both of vector particles and Icon protein. The infected 293 cells are lysed in a few days and therefore should not be capable of forming disseminated nodules. These results suggest that infected 293 cells might be used for efficacious and safe delivery of the Icon.
3. TheraCyte chambers can be used to culture Icon-producer CHO and NIH3T3 cells. When the chamber is implanted in SCID mice carrying a human prostatic tumor, the cells secrete Icon protein into the blood for about one month, resulting in inhibition of tumor growth. The cells do not escape from the chamber and therefore cannot form disseminated nodules. These results suggest that the chambers might be efficacious for extended periods by periodic replacement with new chambers containing fresh Icon-producer cells.
4. Intravenous injections of purified Icon protein, or intratumoral injections of an adenoviral vector encoding the Icon, into SCID mice carrying human prostate tumors resulted in long-term regression of the tumors without associated toxicity.
5. Intravenous injection of radiolabeled Icon protein into TRAMP mice carrying spontaneous mouse prostatic tumors resulted in intense labeling of tumors containing viable cells.

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APPENDICES

No appendices.